Journal of Medical Virology 59:451–455 (1999)

Anti-HCV RIBA/LiaTek Reactivity and HCV Genotype in EIA-Negative Patients With Viremia

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Individuals infected with hepatitis C virus (HCV) usually produce anti-HCV antibodies detectable by enzyme immunoassay (EIA); however, in certain viremic cases this antibody does not appear. To investigate whether anti-HCV in these cases is detectable by Western blot (WB), 38 HCV RNA positive/anti-HCV EIA-negative sera were tested by RIBA 3.0 or LiaTek III. The HCV genotypes (INNO-LiPA) were analyzed to determine whether the variance in these genotypes can be the reason for the late, weak antibody production or its absence. As the control group, 282 EIA-positive/HCV RNA-positive patients were examined. A single band reactivity of various intensities by RIBA or LiaTek was observed in 16/38 EIA negative sera. Positive results with NS3 were detected in 4 sera and weak positive (+/–) with core, NS3, and NS5 in 5, 6, and 1 sera, respectively. In 3 cases with anti-NS3, the seroreversion was observed in follow-up. The distribution of genotypes in anti-HCV-negative versus anti-HCV-positive groups was: 1b alone, 50.0% vs. 78.0%; 3a alone, 13.2% vs. 15.6%; and mixed (1b+3a), 36.8% vs. 5.0%, respectively. The follow-up studies showed that viremia was lost spontaneously in 12/35 patients. In some patients infected with two genotypes, the spontaneous loss of the 3a genotype was observed. The study showed that WB tests are useful for serological confirmation of HCV infection in some EIA negative/HCV RNA-positive patients but, because seroreversion may occur, sequential sera samples should be tested. No unusual HCV genotype was detected in anti-HCV-negative/HCV RNA-positive cases, but the frequency of mixed infection with the 1b+3a genotypes in this group was found to be higher than that in anti-HCV-positive hepatitis patients. ***J. Med. Virol. 59:451–455, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: HCV genotypes; anti-HCV negative hepatitis C; EIA; RIBA; LiaTek

INTRODUCTION

Routine diagnosis of hepatitis C virus (HCV) infection and blood donor screening is carried out by enzyme immunoassay (EIA), which detects antibodies to viral proteins. Viremia can be detected only by molecular biology methods such as the reverse transcription-polymerase chain reaction (RT-PCR). HCV RNA usually occurs together with anti-HCV, but occasionally without it [Sugitani et al., 1992; Wang et al., 1994; Schmidt et al., 1997]. The antibody is not present during the preseroconversion "window period," which usually lasts for 50–190 days after infection. This period may last longer if the immunological status of the infected person has been impaired [Gruber et al., 1993] and sometimes in individuals without any evidence of immunological abnormalities [Panigrahi et al., 1994; Kao et al., 1996; Cerino et al., 1997].

HCV has been classified into several types and subtypes, which differ in immunogenicity [Stuyver et al., 1993; Alonso et al., 1994; Berg et al., 1995; Preston et al., 1995; Dhalival et al., 1996]. To obtain more information concerning the EIA-seronegative/RNA HCV-positive patients, sera were tested for anti-HCV by Western blot (WB) tests and the HCV genotype distribution in this group was compared with that of EIA-seropositive chronic hepatitis C patients.

Grant sponsor: Polish Committee of Sciences; Grant number: 4P05A13414.

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Accepted 16 April 1999

TABLE I. Comparison of RIBA3.0 Reactivity Between EIA-Negative/RNA-Positive Patients and the Control Group (EIA Positive/HCV RNA Positive)

EIA 3.0 (Number of patients)	Number of patients with positive or weak positive reactivity with various number of peptides present in RIBA test				
	Four band	Three band	Two band	One band	Negative
Negative ($N = 38$)	0	1[a]	4[b]	11[c]	22
Positive (Control group) ($N = 45$)	38	5	2	0	0

EIA, enzyme immunoassay; HCV, hepatitis C virus.
[a]+/– NS3, NS4, NS5.
[b]Two cases +NS3, +/–NS4; 1 case +/–NS3, NS5; 1 case +/–NS3, E2.
[c]Two cases + NS3; 5 cases +/– core, 3 cases +/–NS3; 1 case +/–NS5.

MATERIALS AND METHODS

Sera from 522 EIA 3.0 anti-HCV-positive and 231 EIA 3.0-negative patients with chronic hepatitis were tested for HCV RNA by RT-PCR. In 75 of 231 EIA-negative sera, HCV RNA was detected, but only 38 fulfilled the following inclusion criteria: (1) anti-HCV by EIA 3.0 (examined by at least two EIA tests from different manufacturers) negative for at least 6 months after initial HCV RNA result; (2) no other reasons for hepatitis; (3) no obvious immune defect present. Infections with hepatitis A virus (HAV), hepatitis B virus (HBV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), obstructive jaundice, alcoholic liver disease, and autoimmune hepatitis (anti-nuclear, anti-mitochondrial, anti-smooth muscle, anti-liver-kidney microsomal 1 antibodies) were excluded, as well as some causes of immune defects such as HIV infection, dialysis, or steroid therapy. None of the patients had underlying systemic disease or hemophilia; no intravenous drug user was included and none had been transfused. The probable source of infection was surgery in 10/38 patients, hospitalization in 5/38, other iatrogenic contact in 7/38, and unknown in 16/38 patients. Acute hepatitis was diagnosed in 16 cases, and in the remaining 22, chronic hepatitis was diagnosed on the basis of elevated levels of aminotransferases for at least 6 months preceding the first HCV RNA detection. The alanine aminotransferase (ALT) level was at least twice the upper limit of normal in all patients studied.

Sera of all the patients were tested for anti-HCV by RIBA 3.0 and/or LiaTek HCV III and for HCV genotypes. The control groups for WB testing and for HCV genotypes consisted of 45 and 282 EIA 3.0 anti-HCV-positive/HCV RNA-positive chronic hepatitis C patients, respectively.

Serological Tests

The third generation of EIAs (Abbott, Organon Teknika, Ortho Diagnosis, Sanofi Diagnosis Pasteur) and WB tests (RIBA 3.0, Ortho Diagnosis and/or LiaTek HCV III, Organon Teknika) were used. The WB-based tests were carried out on the latest serum sample.

HCV Detection and Genotyping

RNA was isolated according to Chomczyñski and Sacchi [1987]. HCV RNA detection was carried out by RT and PCR amplification according to Lazizi et al. [1992]. The positive results were confirmed by performing the test with the different primer sets complementary to the 5′ noncoding (5′ NC) region [Stuyver et al., 1993] and/or Amplicor HCV test (Roche). HCV genotyping was done using the Line Probe Assay INNO-LiPA HCV (Innogenetics, Belgium). RNA for genotyping was isolated from the serum sample not used previously for HCV detection. To avoid potential contamination by PCR products, the pre- and post-PCR procedures were performed in different rooms with all other recommended precautions. In 35 persons the follow-up studies of HCV RNA were conducted and in 9 the HCV genotyping was undertaken.

RESULTS

HCV RNA was detected in 443/522 (84.9%) anti-HCV-positive patients and in 75/231 (32.5%) anti-HCV-negative patients. Thirty-eight anti-HCV-negative/HCV RNA-positive patients fulfilled the inclusion criteria (see Material and Methods) and were investigated further for anti-HCV in WB tests and for HCV genotype.

The results of supplementary tests (RIBA 3.0 and/or LiaTek III) in the EIA negative/RNA positive sera and in the control group are given in Table I. In four sera the reactivity to NS3 (in two additional +/– NS4) and in 12 others, a weak (+/–) reactivity to NS3 (6 cases), core (5 cases), and NS5 (1 case) region peptide, was detected. In 22 patients, no reactivity to any peptide by RIBA and LiaTek was found. In the control group of 45, the EIA-positive/RNA-positive hepatitis C patients the RIBA results were positive. The reactivity with 4, 3, or 2 peptides was found in 38, 5, and 2 patients, respectively.

A comparison of the HCV genotype frequency in the patients and the control group is shown in Table II. The distribution of genotypes was: 1b alone, 50.0% vs. 78.0%; 3a only, 13.2% vs. 15.6%; and mixed (1b+3a), 36.8% vs. 5.0%, respectively. The HCV genotypes distributions in the seronegative patients with acute and chronic hepatitis were similar.

Follow-up study of HCV RNA presence in the serum samples obtained from 35 EIA-negative patients was carried out. Samples were tested between 1 and 15 months after initial test and, in three patients, the third sample was tested within 52 months. In 12 pa-

TABLE II. HCV Genotype Frequency in EIA-Negative Patients and in the Control Group

Anti-HCV (EIA 3.0)	Clinical manifestation	Number of patients infected with HCV genotype (%)				
		1b alone	Mixed 1b + 3a	3a alone	2a	4
Negative[a] (N = 38)	Chronic hepatitis (N = 22)	12	7	3	0	0
	Acute hepatitis (N = 16)	7	7	2	0	0
	Total (Acute + chronic)	19 (50.0%)	14 (36.8%)	5 (13.2%)	0	0
Positive[a] Control group (N = 282)	Chronic hepatitis	220 (78.0%)	14 (5.0%)	44 (15.6%)	1 (0.4%)	3 (1.0%)

EIA, enzyme immunoassay; HCV, hepatitis C virus.
[a]The difference in genotype frequency is statistically significant for 1b genotype alone chi^2 = 139,0 (P < .001) and for mixed 1b + 3a infection chi^2 = 43.07 (P < .001).

tients, HCV RNA was not detected in the follow-up sample: in 2 (one infected with 1b and the other with 1b+3a), seroconversion by EIA 3.0 occurred simultaneously with viral clearance. In 2 others infected with 1b+3a genotypes, a weak, one band reactivity with core or NS5 was observed simultaneously with the loss of HCV RNA. The patients were not available for further follow-up.

HCV RNA in the second serum sample was detected in 23 cases. In 9, HCV genotyping was conducted (Table III). In 3 patients, the same HCV genotype was detected in both samples. In 6 others, infected initially with two genotypes (1b+3a), the only genotype detected in the serum sample after 1–5 months was 1b. In 7 patients, anti-HCV of various intensity was detected in the follow-up. The result of EIA 3.0 was positive in 1 patient, the single band reactivity with NS3 in RIBA 3.0 in 3, a weak (+/–) reactivity with NS3 in 2, and with core protein by LiaTek in 1 patient. In 3 patients (ML, San, Ma) the seroreversion was observed and anti-HCV was not detected in the follow-up sample.

DISCUSSION

Much data have been published showing the diagnostic importance of detecting HCV RNA in the sera of anti-HCV-negative patients with hepatitis [Panigrahi et al., 1994; Cerino et al., 1997; Suzuki et al., 1997]. We studied a group of well-selected patients with HCV viremia in whom the anti-HCV was not detected for at least 6 months before they entered the study. The negative results by EIA were confirmed by a test from another manufacturer, because it is known that the tests can differ in sensitivity. The patients had no obvious symptoms and causes of an immune defect and all other probable reasons of hepatitis were excluded. The positive results by PCR in all the patients were confirmed by a second PCR (different set of primers from 5′UTR region and/ or Amplicor HCV test) and by using a different serum sample for HCV genotyping.

Serological studies of the viremic patients using WB-based tests revealed a weak, single band reactivity in some patients. According to the manufacturers' recommendations, these results should be interpreted as indeterminate or negative. In the literature, there are no data similar to ours because, in general, the WB tests are used only to exclude false-positive EIA results, but not to detect antibodies in EIA-negative cases [Schmidt et al., 1997]. In our study, a single band reactivity was observed only in three cases for a short period of time, with the subsequent seroreversion. The importance of the full or partial seroreversion in HCV infection was stressed by Lefrere et al. [1997].

Our study shows that to confirm HCV infection using serological tests, in some patients sequential sera samples should be tested not only by EIA but also by WB-based tests. Even a single band reactivity by WB may be important for confirmation. In most patients, however, the results are negative and HCV RNA remains the only marker of infection. According to Cerino et al. [1997], such patients do not produce antibodies to highly immunoreactive second envelope (E2) polypeptide, not included in the current anti-HCV assays. Troisi and Hollinger [1997] found that, in some HCV RNA-positive/EIA-negative patients, antibodies are sequestered in circulating immunocomplexes and can be detected after dissociation of the complexes.

A single band reactivity to the NS3 protein observed in our patients might suggest an infection with genotypes other than HCV 1 [Dhalival et al., 1996]. Because HCV genotypes differ in immunogenicity [Alonso et al., 1994; Dhalival et al., 1996], we compared the genotype prevalences in anti-HCV-positive and -negative hepatitis patients. The viral mechanism of the anti-HCV negativity was examined by Kao et al. [1996], who sequenced the NS3 coding region of the HCV isolates from 10 EIA-negative individuals infected with 1b genotype. Their results indicate that the seronegativity could not be attributed to the variations of those immunodominant epitopes. In our study, almost a half of the patients had the mixed 1b+3a infection. The proportion between the numbers of copies of the virus genotypes that participated in the mixed infection is not known. INNO-LiPA can detect a genotype, if its threshold is 1% of the PCR product [Maertens and Stuyver, 1997]. It is possible that, in a mixed infection, the less immunogenic 3a genotype predominates. According to Dhalival et al. [1996], the antibody signals

TABLE III. Follow-up of HCV Genotypes in Anti-HCV-Negative/HCV RNA-Positive Patients

Patient no.	Name	Clinical diagnosis	HCV genotype		Anti-HCV follow-up
1	MI	Chronic hepatitis	1b	First sample	EIA 3.0 (–), RIBA (–) but +/– only NS3
			1b	24 months later	Negative
2	San	Chronic hepatitis	1b	First sample	EIA 3.0 (–), RIBA ind (only NS3)
			1b	18 months later	Negative
3	Sz	Chronic hepatitis	1b	First sample	Negative
			1b	6 months later	Negative
4	Ko	Chronic hepatitis	1b/3a	First sample	Negative
			1b	5 months later	EIA 3.0 (–), LiaTek (–) but +/– with core
5	Ry	Chronic hepatitis	1b/3a	First sample	Negative
			1b	6 months later	EIA 3.0 (+), LiaTek (–)
6	De	Chronic hepatitis	1b/3a	First sample	Negative
			1b	6 months later	EIA 3.0 (–), RIBA ind (only NS3)
7	Ma	Acute > chronic hepatitis	1b/3a	First sample	EIA 3.0 (–), RIBA ind, LiaTek ind (only NS3)
			1b	9 months later	Negative
8	Gra	Acute > chronic hepatitis	1b/3a	First sample	EIA 3.0 (–) RIBA, LiaTek (–) but +/– NS3
			1b	10 months later	EIA 3.0 (+), RIBA ind (only NS3)
9	Kra	Chronic hepatitis	1b/3a	First sample	Negative
			1b	1 month later	Negative

HCV, hepatitis C virus; EIA, enzyme immunoassay; ind, indeterminate.

from the genotype 1 infection were up to 4.5 times stronger than those from the genotypes 2 or 3.

The high frequency of mixed infections in our patients is difficult to explain. The exacerbation of chronic infection (caused by superinfection with the second genotype), considered by Kao et al. [1994] to be a cause of mixed infection, can also be taken into account in some of the patients. In patients with hemophilia, the high frequency of mixed infection was attributed to the contact with plasma concentrates from many donors [Preston et al., 1995]. There were, however, no patients with hemophilia or intravenous drug users in the group of patients investigated. On the other hand, almost half of the 38 patients had been admitted to hospital some months before the hepatitis symptoms occurred. The present study reveals that in some cases, sporadic hepatitis C may be related to infection with more than one genotype. This can also be the explanation of the high frequency of mixed infections observed in the hepatitis C patients in an epidemic area in Japan [Yamada et al., 1994].

In addition, our follow-up observations show that, during the infection one genotype may be lost, in some patients simultaneously with seroconversion. The 3a genotype in those patients is more sensitive to spontaneous loss than 1b. This finding is in agreement with the observation that, in 3a-infected persons, interferon treatment is successful more often [Berg et al., 1995] than in 1b-infected patients.

REFERENCES

Alonso C, Qu D, Lamerin JP, de Sanjose S, Vitvitski L, Li J, Berby F, Lambert V, Cortey ML, Trepo C. 1994. Serological responses to different genotypes of hepatitis C virus in France. J Clin Microbiol 32:211–212.

Berg T, Konig V, Kuther S, Heuft HG, Witmann H, Hopf U. 1995. Prognostic importance of hepatitis C genotypes for the responsiveness to interferon-alpha. Gastroenterology 33:426–430.

Cerino A, Bissolati M, Cividini A, Nicosia A, Esumi M, Hayashi N, Mizuno K, Slobbe R, Oudshoorn P, Solini E, Asti M, Mondelli MU. 1997. Antibody responses to the hepatitis C virus E2 protein: relationship to viremia and prevalence in anti-HCV seronegative subjects. J Med Virol 51:1–5.

Chomczyñski P, Sacchi N. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Dhalival SK, Prescott LE, Dow BC. 1996. Influence of viremia and genotype upon serological reactivity in screening assays for antibody to HCV. J Med Virol 48:184–190.

Gruber A, Norder H, Magnius L, Rotzen M, Rubio C, Grillner L, Bjorkholm M. 1993. Late seroconversion and high chronicity rate of hepatitis C virus infection in patients with hematological disorders. Ann Oncol 4:229–234.

Kao JH, Chen PJ, Yang PM, Lai MY, Wang TH, Chen DS. 1996. Absence of extensive genetic heterogeneity of hepatitis C virus in antibody-negative chronic hepatitis C. J Med Virol 49:87–90.

Kao JH, Chen PJ, Yang PM, Wang TH, Chen DS. 1994. Mixed infections of hepatitis C virus as a factor in acute exacerbation of chronic type C hepatitis. J Infect Dis 170:1128–1133.

Lazizi V, Bifassi E, Pilot J. 1992. Detection of hepatitis C virus sequence in sera with controversial serology by nested polymerase chain reaction. J Microbiol 30:9312–9314.

Lefrere JJ, Guiramand S, Lefrere F, Mariotti M, Aumont P, Lerable J, Petit JJ, Girot R, Morand-Joubert L. 1997. Full or partial seroreversion in patients infected by hepatitis C virus. J Infect Dis 175:316–322.

Maertens G, Stuyver L. 1997. Hepatitis C virus geno- and serotypes: typing methods and clinical application. In: Decker R, Troonen H, editors. Hepatitis C. Abbott Diagnostics Educational Services. p. 107.

Panigrahi AK, Nanda SK, Dixit RK, Acharya SK, Zuckerman AJ. 1994. Diagnosis of hepatitis C virus-associated chronic liver disease in India: comparison of HCV antibody assa6y with polymerase chain reaction for the 5′ noncoding region. J Med Virol 44: 176–179.

Preston FE, Jarvis LM, Makris M, Philp L, Underwood JC, Ludlam CA, Simmonds P. 1995. Heterogeneity of hepatitis C virus genotypes in hemophilia: relationship with chronic liver disease. Blood 85:1259–1262.

Schmidt WN, Wu P, Cederano J, Mitros FA, LaBrecque DR. 1997. Surreptitious hepatitis C virus infection detected in the majority of patients with cryptogenic chronic hepatitis and negative HCV antibody tests. J Infect Dis 176:27–33.

Stuyver L, Rossau R, Wyseur A, Duhamel M, Vandergorght B, van Heuverswyn H, Maertens G. 1993. Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. J Gen Virol 74:1093–1102.

Sugitani M, Inchauspe G, Shindo M, Prince AM. 1992. Sensitivity of serological assays to identify blood donors with hepatitis C viremia. Lancet 339:1018–1019.

Suzuki F, Chayama K, Nakajiama T, Kawasaki T, Kumada H, Yoshimi T. 1997. Case report: clinical and virological analyses of a patient positive for hepatitis C virus-RNA by branched DNA assay but negative for anti-hepatitis C virus antibodies. J Gastroenterol Hepatol 12:869–873.

Troisi CL, Hollinger FB. 1997. Detection of antibodies to hepatitis C virus in seronegative patients using an immune complex dissociation assay. J Viral Hepat 4, 383–6.

Wang Y, Tao QM, Zhao HY, Tsuda F, Nagayama R, Yamamoto K, Tanaka T, Tokita H, Okamoto H, Myiakawa Y. 1994. Hepatitis C virus RNA and antibodies among blood donors in Beijing. J Hepatol 21:634–640.

Yamada M, Fukuda Y, Imoto M, Koyama Y, Hayakawa T, Takayama T, Nagura H. 1994. High rate of mixed genotypes of hepatitis C in patients of an epidemic area in Japan. J Gastroenterol Hepatol 9:620–623.